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Natural Transformation in River Epilithon

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Natural transformation was demonstrated in unenclosed experiments incubated in river epilithon. Strains of *Acinetobacter calcoaceticus* were transformed to prototrophy by either free DNA (lysates) or live donor cells. The sources of transforming DNA and recipient culture were immobilized on filters, secured to stones, and incubated midstream in the river. The transfer frequency generally increased with temperature. No transfer was detected in the river Taff below 10°C. The age of the recipient culture affected the transformation frequencies in situ but did not significantly affect the transfer frequency on laboratory media. Transformation of recipient cultures which had been incorporated into the natural epilithic biofilm and transformation of the plasmid pQM17 in situ were also demonstrated. This study provides the first direct evidence of natural transformation in situ of bacteria incorporated into an indigenous community.

Natural transformation is a process in which competent cells take up exogenous DNA and incorporate it heritably into their genomes. For a recent review of natural transformation in the environment see reference 21. In recent years there has been increasing concern that horizontal gene transfer events such as transformation could result in the spread of undesirable characteristics (10, 17). Uncertainty as to whether recombinant sequences from genetically engineered organisms might be transferred to indigenous populations has highlighted the need to better understand gene transfer processes in nature (8, 32, 33). Unfortunately, although transformation was described as early as 1928 (12), comparatively little is known about its occurrence or impact in nature.

The ability to take up DNA (competence) may be common in natural populations. Frischer et al. (9) found that 14% of randomly isolated marine heterotrophs were competent to take up homologous chromosomal DNA and that 10% were able to take up a broad-host-range plasmid, pQSR50. However, it is not known whether these organisms express competence in the environment. Several authors have studied natural transformation in laboratory-based microcosm experiments. For example, Graham and Istock (11) examined the transformation of *Bacillus subtilis* in soil microcosms, Stewart et al. (31) studied the transfer of rifampin (RIF) resistance between strains of *Pseudomonas stutzeri* in sediment microcosms, and Paul et al. (22) demonstrated the uptake of plasmid DNA by a marine *Vibrio* strain in marine water and sediment microcosms. These studies clearly demonstrate that natural environments have the potential to support natural transformation. However, as microcosms cannot reflect all the variation that an environment is subject to, in situ experiments are necessary to prove that transformation will occur in these environments.

We have recently shown that natural transformation occurs in river epilithon. An auxotrophic mutant of *Acinetobacter calcoaceticus* was transformed to prototrophy in unenclosed experiments (36). Epilithon provides a suitable environment in which to study gene transfer because the dense, sessile bacterial population and relatively high levels of activity can promote close bacterial interactions (3, 8, 18). *A. calcoaceticus* BD413 was chosen to model naturally competent bacteria in the epilithon because strains of *Acinetobacter* are common in soil and water (6, 14) and because several strains, including BD413, are competent for transformation (14, 15, 34).

In this article we describe natural transformation in three different river systems and examine the effects of temperature, recipient age, and preincorporation of the recipient into the epilithic biofilm on gene transfer.

MATERIALS AND METHODS

Bacterial strains. Spontaneous mutants of *A. calcoaceticus* BD413 (13) were isolated by direct plating on selective media, and where relevant, pQM17, a 7.8-kb plasmid that encodes mercury resistance and that was isolated from river epilithon (25), was introduced by natural transformation to produce the following strains: HGW98(pQM17) (His$^+$ Met$^-$ Hg^r), HGW1521(pQM17) (His$^-$ Met$^+$ Rif^r Sp^r Hg^r), and HGW1510 (His$^+$ Met$^+$ Rif^r Sp^r), where Rif^r is RIF resistance, Sp^r is spectinomycin resistance, Hg^r is mercury resistance, and "–" indicates an auxotroph.

Transformation on agar in the laboratory. The source of transforming DNA was prepared as either an untreated Luria broth (LB) culture or a crude lysate (13, 35). Overnight LB cultures of the recipient (1 ml) and source (1 ml) of DNA were deposited onto separate membrane filters (nitrocellulose pore size, 0.45 μm; 25 mm in diameter) and then placed together so that the recipient and source of DNA were in contact. The mating mixture was incubated on standard plate count agar (PCA; catalog no. CM463; Oxoid) at the relevant temperature (usually 20°C) for 24 h, then removed, resuspended in B22 salts solution (4) containing DNase I (50 μg/ml; Sigma), and enumerated on selective media by drop counts. The precision for the drop count method was ±11% ([standard deviation/mean] × 100).

When lysates were used as a source of DNA, recipients were selected on PCA plus RIF (100 μg/ml) and transformants were selected on S22 (minimal medium supplemented with 14 μg of $HgCl_2$ and 75 μg of RIF per ml) (36). When untreated LB cultures were used as the source of DNA, recipients were selected on PCA plus RIF (100 μg/ml), transformants were selected on SE22 (minimal medium supplemented with 14 μg of $HgCl_2$, 75 μg of RIF, and 300 μg of EDTA per ml, and 0.5% [vol/vol] culture filtrate) (36), and donors were selected on either minimal medium (4) or PCA plus Hg (27 μg/ml) for BD413 or HGW98(pQM17), respectively. The transformation frequency was expressed as the number of transformants per recipient.

Transformation in beaker microcosms. Water was collected from three different rivers: the river Taff (an organically polluted, fast-flowing river in south Wales), the Hillsborough River (a slow-flowing river in south Florida fed by runoff from wetlands), and the Weeki Wachee River (a pristine, medium-flow-rate river from a spring source in Florida). The microcosms consisted of 1-liter beakers containing 400 ml of either sterile (autoclaved 15 min at 15 lb/in^2) or freshly collected river water. Mating experiments were performed as described for matings on agar except that the filters were placed on the surface of a sterile scrubbed slate disc (26), covered with a larger filter (no. 1; Whatman), secured

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with elastic bands, and then placed in the beaker microcosm. Discs were incubated at 20°C for 24 h, and then the filters were retrieved and resuspended in 3 ml of B22 salts solution containing DNase I (50 μg/ml). The numbers of transformants and recipients were estimated as described for matings on agar.

Transformation in situ. In situ matings were performed in triplicate (unless otherwise stated) in the Taff, Hillsborough, and Weeki Wachee rivers. Typical flat stones (pennant stone; about 10 by 15 by 2 cm) were taken from the river Taff, scrubbed to remove the epilithon, and sterilized. As no suitable native stones were available in the Hillsborough or Weeki Wachee rivers, slate discs (26) were used instead. Filters harboring the sources and recipients of DNA were prepared as described for laboratory matings and then transported separately on ice in sterile containers to the river. They were then placed on the surface of a sterile scrubbed stone, covered with a larger filter (no. 1; Whatman), and secured with elastic bands. Stones were placed in a nylon mesh bag (mesh size, 2 by 2 cm), attached by nylon monofilament to a metal stake, and submerged approximately midstream on the bed of the river. After a 24-h incubation, the filters were retrieved and the cells were resuspended in 3 ml of B22 salts solution containing DNase I (50 μg/ml). The numbers of transformants and recipients were estimated as described for matings on agar.

Transformation of cultures incorporated into epilithon. Recipient and donor cultures were incorporated into growing epilithon (in situ). LB cultures (1 ml) were deposited on membrane filters and placed face down on sterile scrubbed stones. They were secured, enclosed in mesh bags, and incubated in the river for 24 h to allow development of a biofilm. The filters were then removed, and the area of the stone where they had been was marked with a diamond marker. The stones with recipients incorporated into the growing epilithic biofilm were then used for in situ matings. Either a filter harboring a fresh lysate or a stone with an incorporated donor culture was placed in contact with the recipient culture incorporated in the epilithon. The sample was reincubated in the river for a further 24 h and then removed and returned to the laboratory in a sterile plastic bag on ice. In the laboratory the area of the stone harboring the recipient was scrubbed with a short, stiff-bristled stencil brush for 3 min in 3 ml of B22 salts solution containing DNase I (50 μg/ml) and the numbers of transformants and recipients were estimated as described for matings on agar.

Effect of growth of the recipient culture prior to mating. Tubes of LB (4 ml) were inoculated with a stationary-phase culture of HGW1521(pQM17) at various times to produce cultures that had been grown for 15 min to 50 h. After incubation, the optical densities were measured at 540 nm and appropriate volumes were filtered to give approximately 10^7 cells per cm^2. These recipients were then transformed to prototrophy by lysates of BD413. Cultures were incubated for 24 h on PCA at 20°C, in B22 salts solution solidified with agar (B22 salts agar) at either 5 or 20°C, or in situ in the Hillsborough River. In situ matings were performed concurrently, but cultures were positioned away from each other to avoid interference.

Effect of the amount of lysate added. HGW1521(pQM17) was transformed to prototrophy in laboratory filter matings as described above but with various amounts of lysate (0.01 to 10 ml). Matings were performed either singly or in triplicate. Three separately prepared lysates of BD413 were compared.

Confirming transformation. Transformant colonies were picked off and subcultured on PCA. They were then screened for secondary characteristics by their ability to grow on selective media and for the presence of plasmids by the method of Kado and Liu (16). Laboratory mutation frequencies were calculated by plating the recipient only on selective media. The limit of detection for in situ experiments was determined by control experiments with recipient cultures but without a source of DNA added. To demonstrate that gene transfer had occurred while the sample was in the river, control experiments were performed by immediately estimating transfer frequencies without incubating samples in situ.

Statistics. Mean values were compared by analysis of variance, before which homogeneity of variance was confirmed by using Bartlett's test (29). Log_{10} transformations were applied to transfer frequencies to give homogeneous variances. Analysis of variance and regression analysis were performed with Minitab version 7.1 (Minitab Inc., State College, Pa.). Minimum significant differences, used to compare individual mean values, were calculated by the Tukey-Kramer method (29) at a P of ≤ 0.05.

RESULTS

Transformation of HGW1521(pQM17) to prototrophy by chromosomal DNA on agar in beaker microcosms and in situ. Recipient strains of *A. calcoaceticus* were shown to acquire an altered genotype following a 24-h incubation with either a crude lysate or an untreated donor culture, both in the laboratory on agar and in unenclosed experiments in the river Taff. No transformants were detected in control experiments in which samples were not incubated but instead were immediately resuspended. Table 1 lists the mean transformation frequencies observed on PCA in the laboratory and in situ in the river Taff. In situ transformation experiments were repeated in two other river systems, the Hillsborough River and the Weeki Wachee River. Transformation was detected in sterile and nonsterile beaker microcosms and in unenclosed experiments for all three rivers (Table 2).

TABLE 1. Transformation and mutation of HGW1521(pQM17) to prototrophy in the laboratory and in situ

Source of transforming DNA	Transformation frequency of HGW1521(pQM17)[a]	
	PCA (laboratory), 20°C	River Taff, 17°C
None	4.90×10^{-9}	2.09×10^{-7}
Lysate of BD413	7.13×10^{-4}	7.94×10^{-4}
BD413 whole cells	9.55×10^{-5}	7.76×10^{-6}

[a] Cultures were incubated for 24 h under the indicated conditions. Frequencies are expressed as numbers of presumptive transformants (colonies growing on selective media) per recipient and are the means of three replicate experiments. The minimum significant difference at which these means could be distinguished was 1.04 log units.

Effect of temperature. Transfer of the chromosomal wild-type histidine gene was detected in the laboratory at temperatures as low as 2°C. The transfer frequency increased slightly with temperature (Fig. 1A). Figure 1B and C show the mean transformation frequencies plotted against the water temperature for experiments performed in situ. The Weeki Wachee River maintained a constant temperature of 24°C all year at the sample site. The river Taff and Hillsborough River varied between 2 and 22°C and between 18 and 30°C, respectively. Both rivers usually varied ±1°C during a 24-h period. No transformation was detected at frequencies significantly higher than the spontaneous mutation frequency in the river Taff at temperatures between 2 and 6°C (during winter). The transformation frequency generally increased with temperature until it was as high as those that could be detected in the laboratory. The highest individual transformation frequency detected in situ was 1.04×10^{-2} in the Hillsborough River at 22°C.

In some cases, a high degree of variation (up to a 100-fold difference in transfer frequencies) between replicate in situ experiments was observed. Further experiments were carried out to determine whether differences in the recipient culture or lysate preparation were responsible for this variation.

Effect of the amount of lysate added. The transformation frequency of HGW1521(pQM17) to prototrophy increased with the amount of lysate added until a saturated frequency of approximately 10^{-3} was reached. Differences between different lysate preparations were observed, but in each case 1 ml of lysate gave a transfer frequency of approximately 7.24×10^{-4}.

Effect of growth of the recipient culture prior to mating. The age of the recipient culture affected the transformation frequencies in situ but did not significantly affect the transfer

TABLE 2. Comparison of transformation frequencies in different river water environments[a]

River	Mean transformation frequency (no. of replicate experiments)[b]		
	Sterile microcosm	Nonsterile microcosm	In situ
Taff	1.10×10^{-4} (1)	2.45×10^{-5} (1)	7.88×10^{-4} (3)
Hillsborough	9.66×10^{-4} (2)	4.62×10^{-5} (4)	1.02×10^{-3} (6)
Weeki Wachee	5.50×10^{-4} (1)	1.00×10^{-3} (1)	2.20×10^{-6} (4)

[a] Transformation of HGW1521(pQM17) to prototrophy was by lysates of BD413. Matings on filters attached to stones were incubated at 20°C for 24 h.

[b] The minimum significant difference at which means could be distinguished was 2.60 log units.

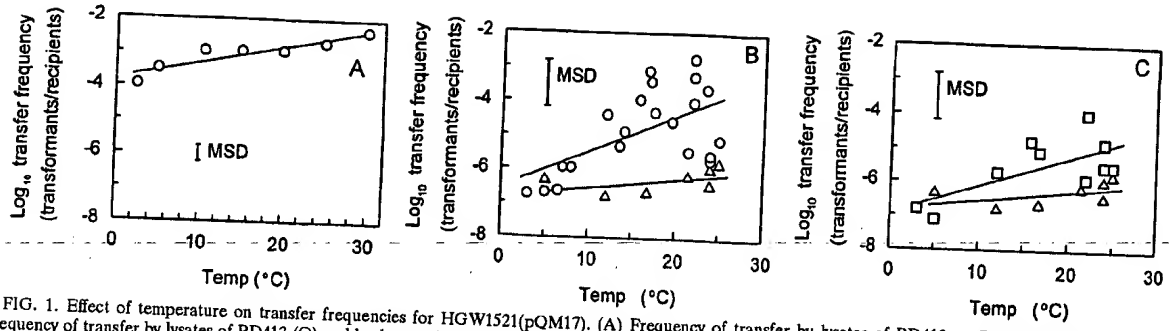

FIG. 1. Effect of temperature on transfer frequencies for HGW1521(pQM17). (A) Frequency of transfer by lysates of BD413 on PCA in the laboratory. (B) Frequency of transfer by lysates of BD413 (O) and background frequency (Δ) in situ. (C) Frequency of transfer by BD413 whole cells (□) and background frequency (Δ) in situ. The background frequency was obtained when HGW1521(pQM17) was incubated in the river without a source of DNA. The river temperature was taken to be the median of the minimum and maximum temperatures recorded. In situ experiments at 20°C or lower were performed in the Hillsborough River or the Weeki Wachee River. Each point represents the mean of three replicate experiments using different cultures incubated in the river on the same occasion. The minimum significant difference (MSD) which could be distinguished between mean transfer frequencies was 0.256 log units in the laboratory and 1.36 log units in situ.

frequency on laboratory media (Fig. 2). Exponential-phase cultures of HGW1521(pQM17) were transformed to prototrophy in situ in the Hillsborough River at transfer frequencies between 7.94×10^{-5} and 1.04×10^{-2}, whereas stationary-phase cultures had transformation frequencies between 1.00×10^{-7} and 2.45×10^{-6} (at 20 to 24°C).

Transformation of *A. calcoaceticus* incorporated into river epilithon. HGW1521(pQM17) incorporated into epilithon in the Hillsborough River was transformed to prototrophy by lysates of BD413 at a frequency of 1.00×10^{-4} ($n = 3$). When whole donor cells were used to transform HGW1521(pQM17) incorporated into epilithon, no transformants were detected above the background frequency of 1.06×10^{-6} ($n = 3$).

Transformation of the plasmid pQM17. Recipient cultures (HGW1510) were transformed to mercury resistance by plasmid pQM17. Mean transfer frequencies ($n = 3$) of 1.11×10^{-6} and 4.64×10^{-7} on PCA at 20°C in the laboratory were observed when plasmid DNA was added as lysates or whole cells, respectively, of HGW98(pQM17). The frequency of mutation of HGW1510 to mercury resistance in the laboratory was $<10^{-9}$ ($n = 3$). Transformation of pQM17 occurred in situ in the Hillsborough River at mean transfer frequencies ($n = 3$) of 3.07×10^{-7} from lysates and 1.26×10^{-7} from whole cells. The frequencies of presumptive transformant colonies were not significantly different from the background frequency of 3.33×10^{-7} ($n = 3$) observed when the recipient only was incubated in the river. However, all of the presumptive transformant colonies tested ($n = 5$) were shown to contain pQM17, but none of the colonies formed on control plates contained the plasmid. These results confirm that transformation of plasmid pQM17 did occur in situ. No transformants were detected when HGW1510 was incorporated into the epilithon.

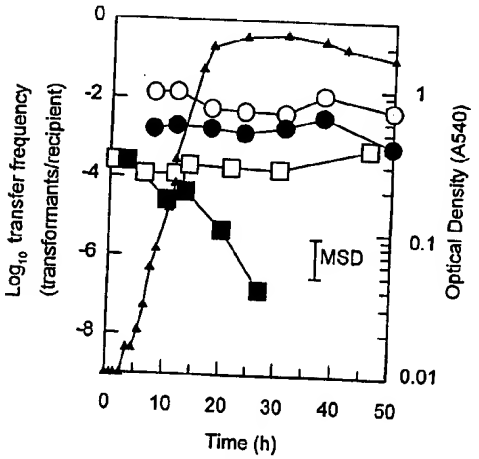

FIG. 2. Effect of culture age on transformation. Cultures of HGW1521 (pQM17) grown between 15 min and 50 h were transformed to prototrophy by lysates of BD413 in the laboratory at 20°C on PCA ( ) at 20°C on B22 salts agar (O) and at 5°C on B22 salts agar (□) or in the Hillsborough River (■) at ~23.5°C. The optical density of the recipient cultures was measured at 540 nm (▲). MSD, minimum significant difference.

DISCUSSION

The aim of this study was to demonstrate that transformation could occur in a natural environment and to examine factors that affect this process. Transformation in a variety of aquatic microcosms has been reported (7, 22, 23, 30, 31). However, this study is the first to report transformation in situ of bacteria incorporated into river epilithon. The differences between river water microcosms and in situ experiments shown in Table 2 demonstrate that the laboratory microcosms do not accurately reflect the environmental conditions. The high degree of variability between replicate experiments performed in situ reflects the dynamic and highly variable nature of the environment, e.g., uncharacterized natural populations and temporal changes in conditions such as temperature, illumination, chemical composition, and flow rates. These results highlight the importance of performing in situ experiments, as controlled laboratory and microcosm experiments do not exhibit the range of responses observed in situ. Control experiments omitting the incubation stage proved that transfer had occurred only during the incubation period. This was important because in some cases gene transfer can occur on selective media (28, 35, 36). The background frequencies observed when no source of DNA was added were presumably due to spontaneous mutation, transfer from the indigenous population, or growth of indigenous organisms. These frequencies represented the limit of detection.

Some transfer frequencies observed in situ were higher than those observed on complex laboratory media. In the laboratory, the transformation of *A. calcoaceticus* did not require growth of the recipient and higher frequencies were observed on B22 salts agar than on complex media (35). Lorenz and

Wackernagel (20) have also demonstrated that transformation may be stimulated by nutrient limitation in the chemical environment provided by a soil extract. The presence of the natural community has been shown to have mixed effects on transformation. The ambient community either reduced the frequency of transformation of a marine *Vibrio* strain or had no effect in water column microcosms (22). It reduced the transformation frequency of *P. stutzeri* (30) and prevented transformation of *Vibrio* strains (22) in sediment microcosms. In this study, transformation of *A. calcoaceticus* was not inhibited by indigenous organisms.

The transformation frequency in situ generally increased with temperature. A strong linear relationship between temperature and conjugation frequency was demonstrated in similar experiments with river epilithon (5). However, there was not an optimal temperature for transformation of the type commonly seen in conjugation experiments (4). These results suggest that gene transfer could be more prevalent in regions with warmer climates and during the summer months of regions with colder climates. Temperature did not have as great an effect on transformation in laboratory experiments on agar. Transformation was detected at temperatures as low as 2°C on agar but was not shown to occur below 10°C in the river. Rochelle et al. (25) reported similar transfer frequencies for cell-to-cell transformation of pQM17 at 12 to 40°C in the laboratory. However, they described much lower transfer frequencies at 4 and 9°C. Analysis of covariance showed a significant correlation ($P < 0.05$) between transfer frequency in situ and temperature for both lysates and whole cells (Fig. 1B and C). The linear fit was not tight ($r = 0.584$ for lysates and $r = 0.779$ for whole cells), suggesting that other unknown factors are also involved in determining transformation frequencies. The high degree of variation observed between replicates in the river might be expected in experiments involving uncontrollable natural factors. However, experiments with recipients grown for different lengths of time prior to mating suggested that the variation may be partly due to differences in recipient culture age. This contrasts with experiments in the laboratory, in which no differences were observed between differently aged cultures. In previous experiments, the time taken for cell-to-cell transformation to occur was also affected by culture age, although the same maximum frequency was eventually reached (35).

The effect of increasing amounts of lysate on transfer frequencies may reflect the availability of transforming DNA. Large quantities of DNA are produced and rapidly turned over in aquatic environments (24), although how much of this is available for transformation is unknown. Lorenz et al. (19) have shown that *A. calcoaceticus* and *B. subtilis* release transforming DNA during growth. In the environment, free DNA would be exposed to degradation by nucleases, etc. The more time recipients require to take up DNA (for example, because of differences in culture age), the less DNA may be available for transformation. Hence, persistence and availability of DNA, competence development, and rate of DNA uptake may be key factors determining the likelihood of transformation in natural environments. Several authors have suggested that DNA may be protected from degradation in the environment by being bound to surfaces such as sediments (1, 7, 27, 31). However, such bound DNA may be unavailable for transformation (31). Cell-to-cell transformation is sometimes observed in the presence of DNase I (2, 34, 35); thus, close interaction between whole cells may protect DNA from degradation during transformation. The influence of cell adhesion on DNA availability and competence development is not known but may prove to be important in determining the efficiency of transformation in biofilms.

Experiments in which recipients were preincorporated into the epilithic biofilm allowed cells to be studied in a more natural state. As well as being exposed to all the natural variation of the environment during mating, the recipients had been acclimatized to the river conditions prior to mating, which is not the case in laboratory cultures. Unfortunately, because low recipient counts resulted in a low limit-of-detection, this method was unsuitable for studying transformation events with low transfer frequencies (such as transformation of plasmid pQM17). The lower transformation efficiency observed for plasmid pQM17 DNA compared with the chromosomal genes tested may reflect the size of the DNA fragment transferred. Transformation is generally most efficient with homologous chromosomal DNA, as the fragment can be integrated into the recipient's genome by recombination (13, 21). Plasmid DNA can be recircularized by mismatch repair if two copies of the molecular are taken up. This would allow plasmid DNA to be transferred to a broad range of hosts without homology and may explain the increased transfer efficiency often observed with plasmid multimers (9).

For transformation to occur in the environment, the conditions must facilitate transformation and there must be a source of transforming DNA and competent recipients. There is an abundance of DNA available for transformation in the environment (24). Competent recipients are common and may form up to 16% of the population in some environments (9). The results of this study prove that the aquatic environment of river epilithon is able to support natural transformation. Transformation is likely to be an important mechanism by which genes, including those from recombinant organisms, could be spread through natural populations and is of particular interest in that genes may be transferred even after the donor organism has ceased to be viable.

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